Acrylamide Disrupts the Ontogeny of Neurobehaviour in Albino Rats

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Abstract: The present study aimed to elucidate abnormalities in the ontogeny of sensorimotor reflexes in developing rats after prenatal and perinatal acrylamide or saline intoxication of pregnant rats. Acrylamide was used as an experimental probe to investigate neurobehavioural and morphological changes in developing rats after administration of the toxin to pregnant mothers. Acrylamide was administered to non-anaesthetised pregnant rats by gastric intubation at a dose of 10 mg/kg/day. Rat pups were assigned to one of three groups: Group A, which comprised pups whose mothers were treated with saline (control group); Group B, which comprised pups whose mothers were treated with acrylamide from day D7 of gestation to birth (prenatal intoxication); and Group C, which comprised pups whose mothers were treated with acrylamide from D7 of gestation to D28 after birth (perinatal intoxication). This study has been conducted recently in Beni-Suef University (Egypt) and Kig Saud University (Saudi Arabia) by May 2012. Acrylamide-induced morphological changes (CNS aberration) and neurobehavioural changes (sensorimotor reflex retardation) were studied. The reflexes tested included rooting, forelimb (FL) grasping, hind limb (HL) grasping, surface body righting, air body righting, FL hopping, HL hopping, chin tactile placing and visual placing. These reflexes were tested in newborns in all groups from postnatal day 2 (D2) until reflex maturation. The appearance of select external features was recorded. administration of acrylamide in pregnant albino rats disrupts the ontogeny of sensorimotor reflexes and morphological changes in the CNS of developing albino rats.
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1. Introduction

While a large number of studies have investigated the neurotoxic and carcinogenic effects of acrylamide, data on acrylamide-induced neurobehavioural and teratogenic effects during embryonic and postnatal development of the albino rat are relatively limited. Acrylamide is an industrial agent used in the manufacture of polymers and synthetic organic chemicals. Polymeric acrylamide is used as a filtration and flocculation aid in water treatment and waste processing, mining and paper mills (Seale etal., 2012). Acrylamide is metabolised to glycidamide (Sumner et al., 2001).

Acrylamide exposure at doses as low as 1.0 mg/kg/day leads to developmental retardation and decreased body weight gain (Garey et al., 2005; Allam et al., 2012) while other studies have documented body weight reductions induced by acrylamide administered intrauterinely. The lowest dose reported to cause significant reductions in body weight was 5.0 mg/kg/day (Allam et al., 2011). Body weight loss is the most sensitive indicator of developmental toxicity (Sumner et al., 2001). Acrylamide has also been detected in breast milk and can cross the human placenta to induce toxicity (Allam et al., 2010). The signs of acrylamide toxicity include decreased body weight and decreased food and water consumption. These toxic effects are also associated with a decrease in the lactation index at high doses of acrylamide (Allam et al., 2011).

In previous developmental studies of acrylamide pinnae detachment was not reported; however, we recently demonstrated that acrylamide delays pinnae detachment by approximately 2 days (Allam et al., 2012). The delay in pinnae detachment is intriguing given that the inner ear develops in part from the same embryonic germ cell layer (i.e., the ectoderm) as the outer ear (Sorgel et al., 2002).

The term reflex refers to a stereotyped distinct neural response that occurs largely at the spinal level and evoked by a fixed and usually peripheral stimulus. These peripheral sensory inputs modify various characteristics of animal behaviours. Impaired reflex input can contribute to severe dysfunction during human walking. The appearance and maturation of a number of sensorimotor reflexes are components of an animal's mature motor repertoire, and the expression of these reflexes can be correlated with the

development and maturation of the nervous system (Cassidy et al., 1994).

The ontogeny of sensorimotor reflexes (e.g., visual and tactile orientation, forelimb (FL) and hind limb (HL) hopping and righting) in albino rats follows a rostral-caudal developmental pattern from birth through adulthood. The neurobehavioural evolution of a normally developing rat may be investigated by means of a series of reflex, motor and sensory tests from birth to weaning. The second week following birth is important in the maturation of the abilities assessed via neurobehavioural tests (ten et al,. 2003; Seale et al., 2012). Such tests may serve as useful baseline references to evaluate changes that may be induced by pharmacological and toxicological agents in developing rats and mice (Gold et al., 2000). Smart and Dobbing (1971) studied the effect of early nutritional deprivation on reflex ontogeny and recorded that the development of physical features and reflexes were significantly retarded in malnourished rats.

Acrylamide adversely affects the CNS, FL hang reflex time, open field activity and other behavioural measures in rats; it also produces detectable effects on rotarod performance prior to the occurrence of other observable effects (Garey et al., 2005). These authors suggested that motor deficits may have just been beginning to appear in the rats at the time they were tested on the rotarod. The performance of multi-model negative geotaxis and rotarod tasks requires the involvement of numerous central and peripheral nervous system components. Thus, the effect of acrylamide on these tasks may involve a variety of systems and/or regions, such as muscle strength, response to fatigue and cerebellar function. However, studies that have correlated behavioural abnormalities with early pathological findings and their evolution have been limited (Allam et al., 2011). High doses of acrylamide exposure have been shown to affect the dopaminergic system. However, appropriate functioning of the vestibular system is essential for negative geotaxis and rotarod performance. Acrylamide exposure alters the trajectory of normal ear development, and the righting reflexes also depend upon vestibular function (Smart and Dobbing, 1971). A dose of 15 mg/kg/day acrylamide produced significant decreases in open field activity at postnatal day 21 in female rats only. The same dose also produced significant decreases in the auditory startle response at postnatal day 22 in male and female Sprague-Dawley rats, although the lower dose (10 mg/kg/day) produced no significant effect (Allam et al., 2010).

The present study was designed to determine the teratogenic effects of oral (gavage) exposure of acrylamide monomer to albino rats during pregnancy and lactation on the development of body weight, external features, the ontogeny sensorimotor reflexes and structural changes in different CNS regions.

2. Materials and Methods

Chemicals: Acrylamide (99% pure) and other chemicals were purchased from Sigma Chemical Company (St Louis, MO, USA). All other chemicals used were of analytical grade.

Animals and dosing schedule: The current study has been conducted recently in Beni-Suef University (Egypt) and Kig Saud University (Saudi Arabia) by May 2012. A total of 60 albino rats (*Rattus norvegicus)* were used in this study. A total of 45 mature virgin females and 15 mature males weighing 140-150 g were purchased from the Organization for Vaccine and Biological Preparations (Helwan laboratory farms, Egypt). The animals were marked, housed with 4 individuals per cage and were fed a standard rodent pellet diet manufactured by the Egyptian Company for Oil and Soap. Food and tap water were provided *ad libitum.* Vaginal smears of each virgin female were examined daily to determine the oestrous cycle. Oestrous females exhibited cornified cells in vaginal smears. Mating was conducted via overnight housing of 2 pro-oestrous females with 1 male in separate cages. The presence of sperm in the vaginal smear was used to determine D1 of gestation. Acrylamide was dissolved in distilled water and orally administered to non-anaesthetised pregnant rats by gastric intubation at a dose of 10 mg/kg/day. Pregnant females were observed daily to record the day of birth.

Three groups of animals were labelled as follows:

Group A: Pregnant rats that were given saline (*Control group*).

Group B: Pregnant rats that were given acrylamide from D7 of gestation to birth (*prenatal* intoxicated group).

Group C: Pregnant rats that were given acrylamide from D7 of gestation to D28 after birth (*perinatal* intoxicated group).

Postnatal investigations: The following observations were recorded daily for pups.

a. Body weight
b. The time of fur appearance.
c. The time of ear opening.
d. The time of eye opening.

Sensorimotor reflexes (Cassidy et al., 1994): at birth, each mother was housed with its pups in a large cage in a ventilated room at a constant temperature (25^{o}C) with a 12:12 h light/ dark cycle. A total of 50 to 80 pups from the first generation of each animal were used from postnatal D2 to D28. Testing was conducted in an open field on a 90×60 cm plastic cart with a 6-cm lip around the edge. The cart was

covered with a sheet of plastic that was wiped with ethanol swabs between testing. Each test was conducted on 6 animals beginning on postnatal D2 until the reflex was at the adult level up to D28. A reflex was considered stable when it was expressed at the adult level for 3 consecutive days. Animals were tested 5 times for a given reflex at a given age, and the positive response was recorded (scores of 0/5 to 5/5). The average score was calculated and converted to the percentage of the adult level, and the data are presented as the per cent reflex. The period between the appearance of a reflex and its stabilisation was considered the period of maturation. The animals were brought to a room that was reserved for testing at a temperature of 25^{o}C. All tests were conducted by the same individual to avoid experimental variation. The Statistical Package for the Social Sciences (SPSS for windows version 11.0; SPSS Inc., Chicago) was used for the statistical analysis. Comparative analysis was conducted by paired samples *t*-test according to the general linear model (SPSS, Inc.). Values of $P > 0.05$ were considered statistically insignificant. Values of $P < 0.05$, $P < 0.01$ and $P < 0.001$ were considered statistically significant, highly significant and very highly significant, respectively.

Rooting: The animal was placed on the test surface, and the experimenter formed a cone with the fingers around the snout of the animal. The reflex was considered present when the animal followed the movement of the experimenter as he slowly withdrew his hands.

Grasp: The animal was held in the air, and the sole of the hand or foot was gently touched with the tip of a fine brush. The left and right sides were tested equally. The reflex was considered present when the animal closed the stimulated hand or foot around the brush.

Hopping: The animal was held so that only one hand or foot touched the test surface, and the animal was moved forward as the tested limb dragged on the surface. The left and right sides were tested equally. The reflex was considered present when the animal lifted its limb and hopped in the direction of the movement. The reflex was tested only in the forward direction.

Body righting on the surface: The animal was held in a supine position so that the dorsum of the head and of the trunk was in contact with the test surface. The reflex was considered present when the animal turned on its ventrum or limbs within 15 seconds after release by the experimenter.

Body righting in the air: The animal was held supine in the air at a distance of 30 cm from a soft surface. The reflex was considered present when, upon release, the animal turned in the air and landed on its ventrum or limbs.

C**hin tactile placing:** The animal was held by the posterior half of its body, and the skin of the chin was gently rubbed on the edge of the test surface. The reflex was considered present when the animal lifted one (or both) of the FLs and placed it on the surface.

Visual placing: The animal was held by the tail at 10 cm from the test surface and was slowly brought closer to the test surface. The reflex was considered present when the animal raised its head and extended its arm towards the surface before the arms came in contact with the surface.

Light microscopy: After sacrifice, the cerebrum, cerebellum, brachial spinal cord and lumber spinal cord were immediately cut into 5 mm^3 pieces and fixed in 20% neutral buffered formalin for 24 h. The tissues were washed to remove excess fixative and then dehydrated in ascending concentrations of ethyl alcohol of 70, 80, 90 and 95% for 45 min each, followed by two changes of absolute ethyl alcohol for 30 min each. This was followed by clearing in two changes of xylene for 30 min each. The tissues were then impregnated with paraplast plus (three changes) at 60ºC for 3 h and then embedded in paraplast plus. Sections (4 to 5 µm) were prepared using a microtome, de-waxed, hydrated and stained in Mayer's haemalum solution for 3 min. The sections were stained in eosin for one min, washed in tap water and dehydrated in ethanol as described above. Haematoxylin- and eosin-stained sections were prepared (Abdul-Hamid et al., 2007).

3. Results

The pups in group B were exposed to prenatal acrylamide, while pups in group C were exposed to perinatal acrylamide. Signs of acrylamide toxicity in postnatally treated mothers included ataxia, splayed HLs, weakness of HL muscles and paralysis causing alterations in maternal behaviour. Consequently, the pups of these mothers suffered from poor lactation and malnutrition, particularly those in group C. The time of fur appearance and ear and eye opening was delayed in groups B and C (Table 1). The mean weights of these pups varied between D1 and D28 (Table 2). The pups in group B exhibited marked decreases in body weight gain, whereas the newborns of group C exhibited even greater decreases in body weight gain (Table 2).

Rooting was absent at all postnatal stages for all individuals across all groups between D2 and D28.

FL grasp reflex was absent in all groups at D2. In normal pups, FL grasp was 31.67% at D4 and increased rapidly to 100% by D10. The pattern of the reflex curve indicated high activation of the reflex during development. In group B, FL grasp was 8.33% at D4 and increased to 100% by D15. In group C, it was ~15% at D5 and increased to 100% by D17. Large

fluctuations were observed in the activation of FL grasp in group B (Fig. 1).

In normal pups, HL grasp appeared after FL grasp. HL grasp was 18.33% at D10 and increased to 100% by D16 (Fig. 2). In groups B and C, HL grasp was 11.67% and 6.67%, respectively, at D13 and reached 100% by D19 and D21, respectively. In group C, reflex development in the first two days was very slow (Fig. 2).

FL hopping was 48.33% at D3 in normal pups and increased rapidly to 100% by D8. In groups B and C, the expression of this reflex was 13.33% and 43.33% at D4 and D5, respectively. The reflex increased slowly to 100% by D14 in group B and by D15 in group C. Figure 3 shows the level of significance between the normal and treated groups.

HL hopping reflex was 38.33% at D3 in the normal group, and its expression increased quickly to 100% by D10. In groups B and C, HL hopping was 21.67% and 11.67%, respectively, at D5 and increased slowly to 100% by D15 and D18 in groups B and C, respectively. The pattern of reflex curves of the acrylamide-treated groups revealed abnormalities in groups B and C when compared to the normal group (Fig. 4).

Body righting on the surface is precocious reflex was already 50% at D2 in normal pups and increased quickly to 100% by D6. However, in groups B and C, this reflex was 10% at D2. Its expression increased to 100% in group B and group C by D12 and D13, respectively. The irregularities in the reflex curve indicate weakness in the pups of groups B and C. The difference between normal and treated groups was significant (Fig. 5).

Body righting in the air, in normal pups, this reflex was ~30% at D11 and increased to 100% by D17. The development of the air body righting reflex in treated groups was 16.67% in group B at D15 and 18.33% at D17 in group C. Expression increased slowly to 100% by D20 in group B and by D22 in group C (Fig. 6).

Chin tactile placing expression was 8.33% at D4 and 100% by D11 in normal pups. Expression of this reflex in groups B and C was 21.67% and 8.33%, respectively, by D8 and increased rapidly to 100% in group B at D16 and in group C at D17 (Fig. 7).

Visual placing reflex was 46% at D15 and matured rapidly to 100% by D17 in the normal group. It was 28.33% and 27% in groups B and C at D16, respectively. Its expression increased to 100% by D19 in group B and by D21 in group C (Figure 8).

The brachial spinal cord consists of centrally located grey matter and peripheral white matter. Neurons and glial cells were distributed in the grey matter. The white matter consisted of neuronal axons and some glial (neuroglia) cells (Fig. 9). At D7, normal motor neurons were large in size, with many processes and oval nuclei (Fig. 9a). In group B, most motor neurons appeared small in size. Pyknosis was also noted (Fig. 9b). In group C, motor neuron chromatolysis was observed (Fig. 9c). Between D14 and D28, the motor neurons in individuals in group A increased in size, with well-differentiated chromatin and a distinct thick coat of cytoplasm (Figs. 9d, g & j). The acrylamide-treated groups exhibited chromatolysis and pyknosis in neurocytes (Figs. 9e, f, i & l). However, group B pups had almost normally structured motor neurons at D21 and D28 (Figs. 9h & k).

The histological structure of the lumbar spinal cord is similar to that of the brachial spinal cord; the difference between these regions depended on the degree of neuronal development. The lumbar motor neurons were relatively smaller than the brachial motor neurons at the same age (Fig. 10). At D7, most lumbar motor neurons appeared small and less differentiated in groups B and C when compared to those of group A. Pyknosis and neurocyte chromatolysis were noted (Figs. 10a, b & c). Between D14 and D28, normal motor neurons increased in size in group A pups, while pyknosis and neurocyte chromatolysis were still present in the acrylamide-treated groups (Figs. 10e, f, i & l). Group B displayed improvement in lumbar motor neuron structures at D21 and D28, as was noted in the brachial region (Figs. 10h & k).

In general, during the first four weeks after birth, the cerebellum of the normal and treated pups consisted of grey and white matter. Grey matter was arranged in four layers. These layers included the superficial external granular layer, the molecular layer with few neurons (stratum molecular), the deep internal granular layer (stratum granulosum) and the ganglionic (Purkinje) cell layer that was present between the molecular and the deep internal granular layers. The white matter was composed of corticonuclear projections; it comprised afferent and efferent fibres. In normal pups, the transverse fissures subdivided the cerebellum into groups of folds (Figs. 11 & 12). These fissures migrated vertically along the antero-posterior axis of the brain.

At D7, the folds and fissures were defined in normal and acrylamide-treated pups. The folds were deeper in the normal group than in the treated groups (Figs. 11a, b & d). The normal group fissures were narrow, while in groups B and C, they were wide and crude. The folds had a prominent external granular layer. This layer was thicker in group A than in groups B and C.

At D14, the folds became deep, and all layers were represented in the normal and treated pups (Figs. 11g, h & i). In group A, there was a decline in the

thickness of the external granular layer. The thickness of this layer in groups B and C was greater than that in group A.

At D21, the folds became deeper in the normal and treated groups. In group A, the external granular layer was absent in some regions at this age but in other regions was represented by a single row of cells, while this layer remained well-represented in groups B and C (Figs. 12a, b & c). At D28, the fissure disappeared in group A animals only, while the external granular layer disappeared in the individuals in all groups (Figs. 12g, h & i).

At D7, the normal thin molecular layer was formed between the Purkinje cell rows and the external granular layer (Figs. 11d, e & f). At D14, increased thickness of the molecular layer was observed in all groups; however, this increase was greater in group A pups compared to the treated groups. The number of molecular layer neurons was greater in the normal group than in the treated groups (Figs. 11j, k & l). The molecular layer consisted of several types of cells: smaller round cells that were presumably neuroglia, large round cells in the upper portion of the layer that were presumably stellate cells, large spherical cells in the lower portion of the layer that were presumably basket cells and vertically oriented spindle-shaped cells.

In group A, the molecular layer increased in width at D21 and D28. Stellate cells were well differentiated, and the vertically oriented spindle-shaped cells were still present in the molecular layer. There were fewer vertically oriented spindle-shaped cells in group A than in the other treated groups (Figs. 12d, e & f). Basket cells, which are spherical in shape, were observed in the lower portion of the molecular layer. The molecular layer neuronal density was highest in group A.

At D7, normal Purkinje cells were visible and were arranged in a single row in group A pups, while in the treated groups, these cells were arranged in more than one row. In group A, Purkinje cells appeared large and spherical in shape, with large nuclei. There were many processes that arose from all aspects of the cell body. In groups B and C, Purkinje cells appeared small (Figs. 11d, e & f).

The Purkinje cells of group A animals at D14 were arranged in a single row of large neurons with pear-shaped perikaryon and large nuclei. Cells increased in size and became spindle shaped. The lateral processes disappeared, and the apical processes formed the permanent dendritic trees (Figs. 11j, k & l). In groups B and C, degenerated and pyknotic Purkinje cells were observed. At D21 and D28, Purkinje cells were arranged in a single row in both normal and treated newborns; this row eventually formed the Purkinje cell layer, as shown in Figure 12. This layer lies at the junction of the molecular layer and internal granular layer. Purkinje cells displayed a normal pear shape in group A pups, while in the treated groups, these cells were more spindle-shaped and smaller. Degenerated Purkinje cells were observed more frequently in group C pups than in group B pups.

At D7, the internal granular layer was sparsely populated in group A (Figs. 11a & d) and less defined in groups B and C (Figs. 11b, c, e & f). At D14, the thickness of this layer was varied. It was well differentiated, and granule cells became numerous and aggregated in all groups (Figs. 11g, h, i, j, k & l). The thickness of the internal granular layer increased after the third week. At D28, the normal internal granular layer formed, consisting of closely populated round granule cells. The density of the internal granular layer cells was higher in group A than in groups B and C. These neurons were infiltrated by intercellular spaces known as islands or glomeruli. The neurons of the internal granular layer had large, darkly stained nuclei that were enclosed in a peripheral cytoplasmic coat as shown in Figure 12.

Normal white matter was more differentiated at D14 than D7 (Fig. 11). At D21 and D28, it became well differentiated, and its ramification filled the core of the cerebellar folds (Fig. 12). Haemorrhagic brain damage was observed in cerebellar sections of group C pups at D7 (Fig. 11c) and D28 (Fig. 12i).

At D7, the borders between the cerebral cortical layers were undetectable in both normal and treated pups. The outermost layer of the grey matter lies just below the delicate connective tissue (pia matter) in the outer molecular layer, which was easily defined at this age because it did not contain neurons (Figs. 13a, b & c). The cells of the cerebral cortex in the pups of the normal group were large in size, and their apical dendrites were perpendicular to the pial surface; however, in the treated groups, the cells were small and undifferentiated (Figs. 13d, e & f). At D14, the normal group pyramidal cells assumed the typical shape and increased in number and size when compared to D7. The nuclei of these cells appeared round, large and centrally located (Figs. 13g & j). At D21, the outer molecular layer was sharply defined (Figs. 14a & d). At D28, the normal cells of the cerebral cortex had a spherical or pyramidal perikaryon whose nuclei were large, and neurons were arranged in a regular pattern (Figs. 14g & j). Moving along the length of the present cerebral neurons towards the white matter, cells developed more gradually (Figs. 14a & g). Pathological cases were identified by evaluating many sections from the newborns of the treated groups. In group B, pyknosis was observed at D14, D21 and D28 (Figs. 13k and 14e & k). In group C, chromatolysis of neurocytes was observed (Figs. 13l and 14f & l).

4. Discussion

The present study was designed to study the ontogenesis of sensorimotor reflexes and structural changes in the cerebral cortex, cerebellar cortex and spinal cord of developing rats after maternal acrylamide exposure. The effect of prenatal and perinatal acrylamide exposure was investigated in the developing rat embryo. Water-soluble acrylamide and its metabolite, glycidamide, pass readily through the placenta (Allam et al., 2010) and are distributed in various foetal tissues during gestation (Sumner et al., 2001). Acrylamide is also passed through the mother's milk to pups during lactation (Allam et al., 2011). In addition, acrylamide treatment leads to poor lactation; this presumably results from poor maternal behaviours, which consequently cause postnatal malnutrition in developing pups(Bouet et al., 2004). Thus, group B pups that were exposed to prenatal acrylamide and group C pups that were exposed to acrylamide during the gestation and lactation periods were exposed to malnutrition and other toxic effects of acrylamide.

In the normal group A pups, fur appeared at D9. Fur appearance was delayed in both acrylamide-treated groups, consistent with Gold [9] who demonstrated that acrylamide causes growth retardation due to protein deficiencies resulting from malnutrition during development (Garey et al., 2005). Compared to the normal group A pups, ear opening was delayed in acrylamide-treated pups (Allam et al., 2012). This suggests that acrylamide exposure impaired organogenesis, as reported by Allam et al. (2010). These results are also in agreement with those reported by Garey et al. (2005). Eye opening may also be delayed due to acrylamide exposure, as observed in groups B and C. This effect of acrylamide supports results reported by Sumner et al. (2001), who observed slowed developmental changes in pups following maternal acrylamide exposure.

The newborn pups of acrylamide-treated dams exhibited body weight loss. The mean birth weight of pups was 5.08±0.03 g in group B and 3.88±0.11 g in group C; the mean normal group A pup birth weight was 6.31±0.12 g, a very highly significant difference. Garey et al. (2005) recorded body weight reductions in pups when female rats were exposed to acrylamide during pregnancy. Prenatal weight reduction may be caused by intrauterine acrylamide exposure, which retards the growth of the developing foetus. Body weight has been reported to be the most sensitive indicator of developmental toxicity (Allam et al., 2011). The toxic effects of acrylamide on embryos must be intrauterine in nature because foetuses lack the enzymes required to degrade the toxin once it has entered the blood supply (Allam et al., 2010). In group A pups, body weight increased with age, and the mean body weight was 43.17±0.99 g at D28. In the treated groups, the increase in body weight was quite slow: 26.62±0.62 g and 24.21±1.11 g at D28 in groups B and C, respectively. In the treated groups, acrylamide affects the function of the mammary glands by reducing prolactin levels in animals, thus impairing lactation. It was demonstrated that the main reason for postnatal weight reduction in treated pups was related to changes in maternal behaviours as well as decreased food and water consumption and lactation index.

The expression of each reflex may be dependent on the state of developmental maturation of a specific portion of the CNS and the rate of its development (Cassidy et al., 1994). The retarded development of sensorimotor reflexes in the treated groups represents a feature of acrylamide neurotoxicity that was observed and previously described by Gary et al., (2005). Acrylamide exposure leads to prenatal and postnatal malnutrition through its effects on maternal behaviours (Bouet et al., 2004). Smart and Dobbing (1971) demonstrated that malnutrition retards the development of sensorimotor reflexes. The results of this study demonstrated that acrylamide induces neuropathy and neuronal loss, leading to the behavioural abnormalities reported by Lehning et al. (2002). Cassidy et al. (1994) suggested that sensorimotor reflex expression is mediated by CNS neuronal activity, and therefore the loss of these neurons would have an adverse effect on the expression of these reflexes. Acrylamide impairs synaptic function and neuronal connectivity and the present results indicate that acrylamide caused weakness, ataxia and malformations in the pups in the treated groups (Shaheed et al., 2006). Similar observations were reported by Lopachin et al. (2002).

The rooting reflex was not observed in normal and treated groups, as previously reported by Abdul-Hamid et al. (2007). Rooting is innate in mammals because it enables young individuals to find warmth and a nipple for feeding (Dalia, 2002). As this need decreases, the reflex also decreases, as reported by Cassidy et al. (1994). The disappearance of rooting in rat pups may be due to the inhibition of the subcortical regions responsible for the reflex by the cerebral cortex. The present study revealed that the laminar organisation of the cerebral cortex was not detectable in the rats at D7 & D14 of the experimental timeline.

FL grasping, HL grasping, FL hopping, HL hopping and surface body righting are spinal reflexes (Cassidy et al., 1994). These behaviours appear early in normal pups as the neuronally mediated reflexes (motor neurons) are differentiated by this age, as supported by the present study. Reflex expression occurs after differentiation of the mediating neurons is complete (Dalia, 2002). In normal pups, expression of the spinal reflexes increased regularly to reach mature and well-formed motor neurons. The FL reflexes

appeared and matured earlier than the HL reflexes. The spinal histological sections revealed that development of the spinal brachial regions occurred earlier than the lumbar region (rostral-caudal development). The ontogeny of sensorimotor reflexes from birth through adulthood has been elaborated, and mature characteristics are observed at various ages during rostral-caudal development. In group B, the spinal reflexes were detected late due to weakness resulting from malnutrition and poor behaviour of acrylamide-treated dams (Bouet et al., 2004). In group C, these reflexes were detected later than in groups A and B as a result of the retarded development and differentiation of reflex-mediated neurons. Reflex development in group C was slow and irregular due to decreased activation of neurons, as previously reported by Garey et al. (2005). With increasing age, these reflexes attained 100% maturation due to persisting small neurons. The late appearance and maturation of grasping and hopping in the treated groups resulted from malformations of the limbs and spinal cord. Acrylamide exposure leads to malnutrition, which delays the appearance and maturation of sensorimotor reflexes (Ten et al., 2003). In addition, acrylamide delayed the development of the reflexes and motor neurons. Dalia (2002) reported that the motor neurons of the ventral horn of the rat spinal cord are already well-differentiated at birth; however, the neurons of the dorsal horn are smaller, less differentiated and more densely packed.

The brachial and lumbar motor neurons that were present were well differentiated at D7. Their number and size increased with age; therefore, the spinal reflexes appeared and matured early after neuronal maturation. Brachial motor neurons appeared to be more differentiated than lumbar neurons. Dalia (2002) reported that brachial motor neurons are large in size and number compared to lumbar motor neurons.

The motor neurons of the acrylamide-treated groups were small and less differentiated than those of the normal pups. Between D7 and D28, the normal pups' motor neurons increased in size, although pyknosis and neurocyte chromatolysis were observed. Rats in group B showed some improvement in the structure of motor neurons at D21 and D28 during lactation. Acrylamide exposure impaired motor neuron functions, including motor coordination, motor control and the ability to generate action potentials (Shaheed et al. 2006).

Air body righting is a cerebellar reflex that appears late in the developmental course of normal pups because cerebellum maturation occurs similarly late in development (Cassidy et al., 1994). This reflex is normally detected at D11 and increases regularly to reach 100% at D17. This pattern of reflex development occurs due to the continuous differentiation and maturation of cerebellar neurons. These results parallel those of Allam et al. (2012). Maturation was reported to reach 100% at D22 in the Mongolian gerbil (Cassidy et al., 1994) and at D21 in both the rabbit and guinea pig (Dalia, 2002). In group B, this reflex was detected at D15 and developed to maturation by D20. In group C, it was detected later, at D17, and reached 100% by D22. This reflex developed earlier in group B than group C because the pups of group C were exposed to acrylamide for a longer period of time during lactation. In both treated groups, retardation of reflex development resulted from either late maturation and differentiation of the cerebellar neurons or from high levels of neuronal loss caused by acrylamide. Similar results were reported by Garey et al. (2005).

At D7, the external granular layer of normal pups was thick due to high cellular proliferation rates. Marcelo and Fahad (2002) observed that the anlage fold appears at birth in normal and acrylamide-treated pups and was represented by the external granular layer. This layer forms by cellular migration in an inside-out pattern (Marcelo and Fahad, 2002). These cells proliferate rapidly in the external granular layer and then migrate inward and differentiate into granular neurons in the internal granular layer (Allam et al., 2011).

The cerebellar cortex of the normal rat newborns at D14 consisted of four layers: the superficial external granular layer, the molecular layer, the Purkinje cell layer and the internal granular layer. The external granular layer was present in normal rats at D14 but was thin because the cells had migrated to the internal granular layer. This layer remained represented at D21 by one row of cells or was completely absent in some regions; it disappeared completely at D28 in the rat, D21 in the guinea pig (Dalia, 2002) and D18 in the mouse (Abdul-Hamid et al., 2007). The disappearance of this layer was attributed to the migration of its cells into the neighbouring molecular layer, which in turn, increased in width via resorption of the external granular layer.

In the acrylamide-treated groups, the external granular layer was thin at D7 due to chronic acrylamide exposure in pre- and postnatal pups; this in turn delayed the proliferation of the cells in the granular layer. Marcelo and Fahad (2002) reported that the external granular layer is the external germinal layer that generates the granule cells; the cells then begin to migrate through the Purkinje cell layer to form the internal granular layer. Therefore, the internal granular layer will be affected by aberrations in the external granular layer (Allam et al., 2012).

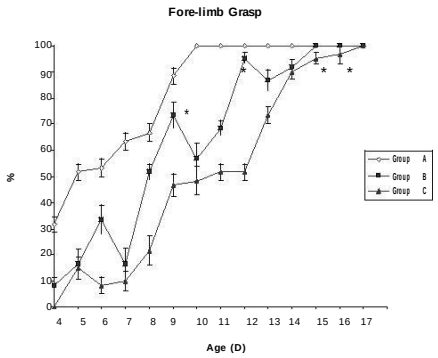

Fig. 1. Percentage response relative to age for FL grasp reflex. The insignificant points are labeled by*

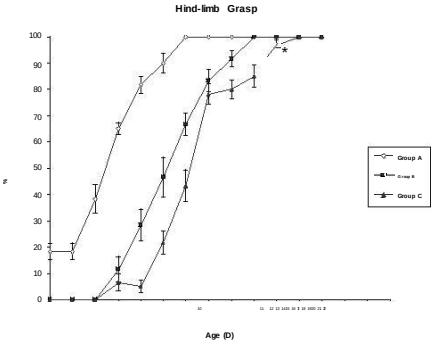

Fig. 2. Percentage response relative to age for HL grasp reflex. The insignificant points are labeled by*

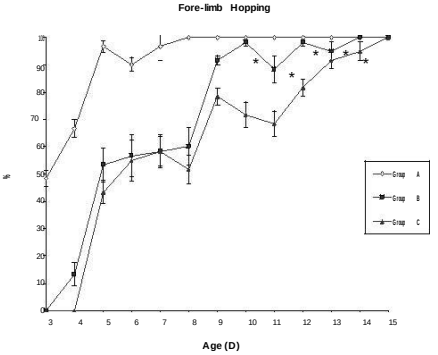

Fig. 3. Percentage response relative to age for FL hooping reflex. The insignificant points labeled by *

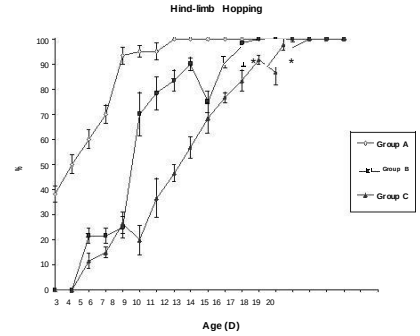

Fig. 4. Percentage response relative to age for HL hooping reflex. The insignificant points labeled by *

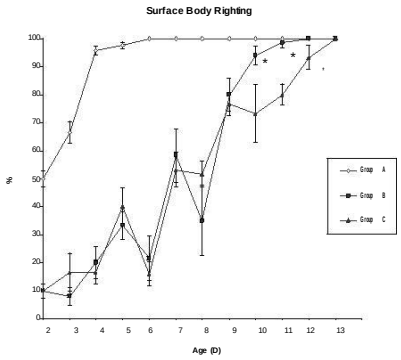

Fig. 5. Percentage response relative to age for surface body righting reflex. The insignificant points labeled by *

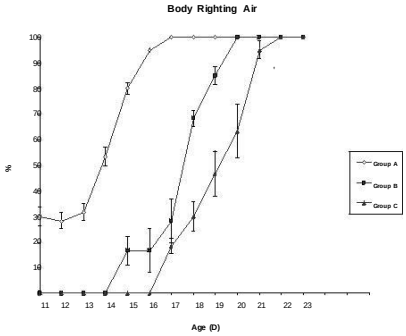

Fig. 6. Percentage response relative to age for air body righting reflex. The insignificant points labeled by *

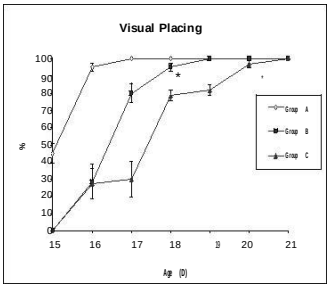

Fig. 7. Percentage response relative to age for chin tactile placing reflex. The insignificant points labeled by *

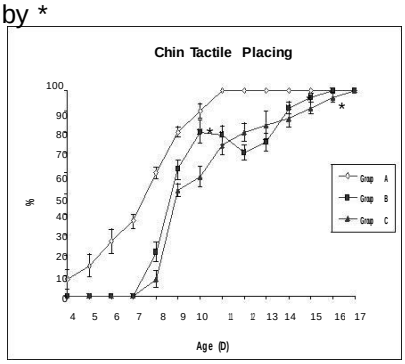

Fig. 8. Percentage response relative to age for visual placing reflex. The insignificant points labeled bystar

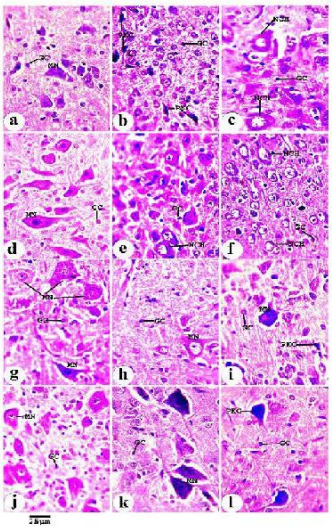

Fig. 9: Transverse sections in the brachial spinal region showing the glial cells (GC), motorneurons (MN), neurocyte chromatolysis (NCH) and pyknosis (PKC). (**a**) normal group at D7, (**b**) group B at D7, (**c**) group C at D7, (**d**) normal group at D14, (**e**) group B at D14, (**f**) group C at D14, (**g**) normal group at D21, (**h**) group B at D21, (**i**) group C at D21, (**j**) normal group at D28, (**k**) group B at D28 and (**l**) group C at D28. Scale bar = 25µm. (**H&E**)

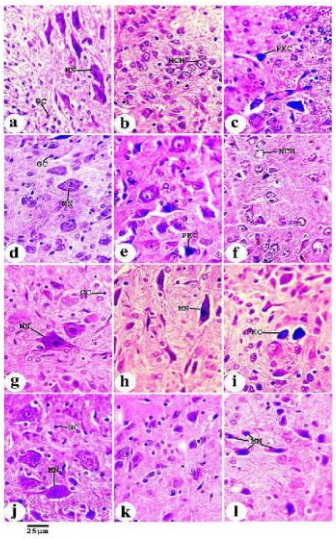

Fig. 10: Transverse sections in the lumbar spinal region showing the glial cells (GC), motorneurons (MN), neurocyte chromatolysis (NCH) and pyknosis (PKC). (**a**) normal group at D7, (**b**) group B at D7, (**c**) group C at D7, (**d**) normal group at D14, (**e**) group B at D14, (**f**) group C at D14, (**g**) normal group at D21, (**h**) group B at D21, (**i**) group C at D21, (**j**) normal group at D28, (**k**) group B at D28 and (**l**) group C at D28. Scale bar = 25µm. (**H&E**)

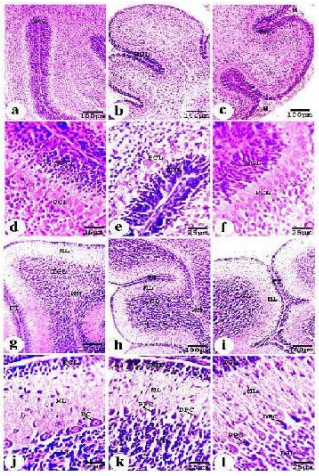

Fig. 11: Sagittal sections in the cerebellar cortex showing the degenerated Purkinje cell (DPC), external granular layer (EGL), fissure (FI), hemorrhage (H), internal granular layer (IGL), molecular layer (ML), Purkinje cell (PC), Purkinje cell layer (PCL), pyknotic Purkinje cell (PPC) and white matter (WM). (**a** and **d**) normal group at D7, (**b** and **e**) group B at D7, (**c** and **f**) group C at D7, (**g** and **j**) normal group at D14, (**h** and **k**) group B at D14 and (**i** and **l**) group C at D14. Scale bar = 100µm in 33a, b, c, g, h & i and 25µm in 33d, e, f, j, k & l. (**H&E**)

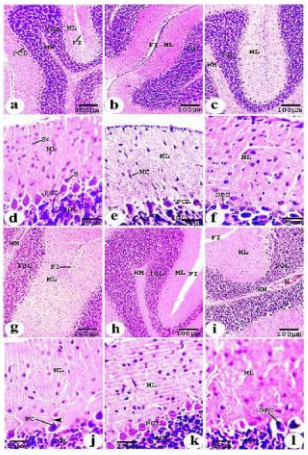

Fig. 12: Sagittal sections in the cerebellar cortex showing the basket cell (B), degenerated Purkinje cell (DPC), external granular layer (EGL), fissure (FI), hemorrhage (H), internal granular layer (IGL), molecular layer (ML), migratory cell (MC), Purkinje cell (PC), Purkinje cell apical dendrites (arrow head), Purkinje cell layer (PCL), stellate cell (SC) and white matter (WM). (**a** and **d**) normal group at D21, (**b** and **e**) group B at D21, (**c** and **f**) group C at D21, (**g** and **j**) normal group at D28, (**h** and **k**) group B at D28 and (**i** and **l**) group C at D28. Scale bar = 100µm in 34a, b, c, g, h & i and 25µm in 34d, e, f, j, k & l. (**H&E**)

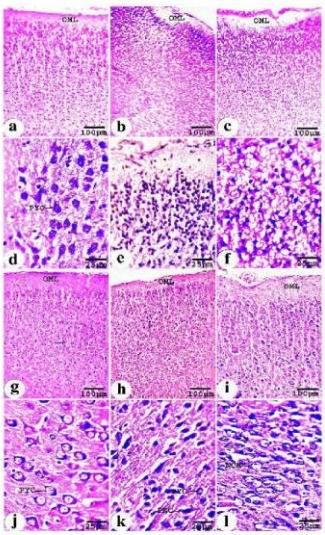

Fig. 13: Sagittal sections in the cerebral cortex showing the outer molecular layer (OML), pyramidal cells distribution (PYC), neurocyte chromatolysis (NCH) and pyknosis (PKC). (**a** and **d**) normal group at D7, (**b** and **e**) group B at D7, (**c** and **f**) group C at D7, (**g** and **j**) normal group at D14, (**h** and **k**) group B at D14 and (**i** and **l**) group C at D14. Scale bar = 100µm in 29a, b, c, g, h & i and 25µm in 29d, e, f, j, k & l. (**H&E**)

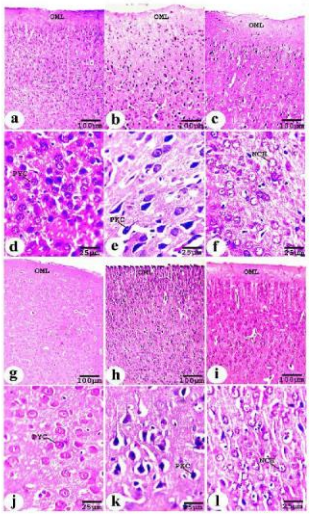

Fig. 14: Sagittal sections in the cerebral cortex showing the neurocyte chromatolysis (NCH), outer molecular layer (OML), pyramidal cells distribution (PYC) and pyknosis (PKC). (**a** and **d**) normal group at D21, (**b** and **e**) group B at D21, (**c** and **f**) group C at D21, (**g** and **j**) normal group at D28, (**h** and **k**) group B at D28 and (**i** and **l**) group C at D28. Scale bar = 100µm in 30a, b, c, g, h & i and 25µm in 30d, e, f, j, k & l. (**H& E**)

Table 1. External features appearance in rat newborns.

Features/Groups	A	B	C
Fur appearing	D9	D11-12	D12-13
Ear opening	D12-13	D15	D15
Eye opening	D14-15	D16-17	D16-17

Table 2. Changes of body weights in rat newborns.

/ Groups			
	.31±0.12	.08±0.13***	.88±0.11***
	.4±0.26	.15±0.08***	.47±0.50**
	.33±0.19	.53±0.16***	.27±0.38**
	.15±0.15	.57±0.11***	.83±0.53***
	.58±0.28	.8±0.13***	.48±0.29***
	0.65±0.23	±0.21***	.23±0.40***
	2.13±0.16	.83±0.27***	.18±0.27***
	3.57±0.1	.85±0.41***	.75±0.31***
	4.27±0.13	.23±0.68***	.13±0.38***
0	6.1±0.15	0.9±0.336***	.9±0.30***
1	7.23±0.13	0.47±0.63***	.33±0.77***
2	7.33±0.07	0.77±0.41***	.85±0.80***
3	8.25±0.09	2.27±0.81***	.27±0.65***
4	9.35±0.15	1.58±0.72***	.33±0.67***
5	0.35±0.08	3.28±0.68***	0.72±0.60***
6	1.60±0.15	5.38±0.42***	0.39±0.34***
7	3.67±0.36	6.93±0.76**	2.93±0.61***
8	3.97±0.36	7.36±0.78***	1.3±0.56***
9	5.22±0.51	9.73±0.40***	1.63±0.60***
0	5.8±0.64	0.68±0.69***	2.47±0.70***
1	7.75±0.60	0.9±0.38***	4.18±0.50***
2	8.47±0.52	1.2±0.51***	6.48±0.47***
3	1.42±0.45	2.85±0.71***	0.12±0.95***
4	4.52±0.35	3.68±0.99***	0.97±0.87***
5	6.07±0.37	3.55±0.77***	1.55±1.13***
6	8.45±0.57	4.65±0.99***	2.40±1.12***
7	9.65±0.62	5.87±77***	3.25±0.64***
8	3.17±0.99	6.62±0.62***	4.21±1.11***

Data are expressed as a mean ± S.E. (N =6)
Values significantly compared to the control newborns; $p^{*} \leq 0.05$, $p^{**} \leq 0.01$and $p^{***} \leq 0.001$.

At D14, this layer was thick in groups B and C because the migration of the cells of this layer was impaired by acrylamide exposure (Marcelo and Fahad, 2002). This suggests that acrylamide exposure delayed cellular migration and differentiation in the granular layer (Allam et al., 2012). At D21, this layer remained represented due to delayed cellular differentiation in the acrylamide-exposed groups.

A thin molecular layer was observed at D7 in the normal and treated groups. At day 14, the molecular layer in normal pups was wide; the width of this layer increased by D21, and neurons were detectable in the layer. It was reported that the width of the molecular layer was maximum at D23. Others have reported that the width of molecular layer depends on the number of neurons and the size of Purkinje cell arborisation. The molecular layer was narrow in groups B and C due to the loss of Purkinje cells as a consequence of malnutrition induced by acrylamide exposure. Also, it was observed that malnutrition induced aberrations in the dendrites of Purkinje cells (Bahgat et al., 2006).

The internal granular layer was composed of sparsely populated cells at D7; this reflects the low rates of cellular migration from the external granular layer. The internal granular layer was not sharply demarcated by underlying white matter until D7 in normal pups. This layer was clearly differentiated by D14. Granule cells were high in number, rounded in shape and occupied the deep region of the internal granular layer and were most often formed after birth. Similar observations were recorded by Bahgat et al. (2006). The granular layer in the treated groups was undifferentiated from the white matter until D7. The internal granular layer was affected by acrylamide-induced aberrations in the external granular layer. Haemorrhagic brain damage was also detected in the cerebellum of acrylamide-treated group C. The chin tactile placing reflex is a cerebral reflex (Cassidy et al., 1994). It appeared early at D4, after spinal reflexes and before cerebellar reflexes. It developed slowly to 100% by D11 in normal rats; thus, the cerebral cortex takes a long time to mature after birth. The development of this reflex was similar in groups B and C. In groups B and C, the chin tactile reflex appeared at D8 and increased to 100% by D16 in group B and by D17 in group C. The late appearance of this reflex was due to the abnormalities induced by acrylamide exposure. The performance of this reflex requires the involvement of numerous central and peripheral nervous system components, including muscle strength and response to fatigue (Garey et al., 2005).

The visual placing reflex is a cerebral reflex, but its expression is related to eye opening (Cassidy et al., 1994). In the normal group, this reflex appeared at D15 and matured at D17. In the acrylamide-treated groups, this reflex was expressed at D16 by 28.33% and 27% in groups B and C, respectively. Expression of this reflex reached 100% by D19 in group B and by D21 in group C. This retardation was produced by acrylamide exposure and led to skeletal muscle weakness and developmental alterations in the offspring. Acrylamide is also known to impair synaptic function and neuronal connectivity (Seale et al., 2012), thereby affecting the expression of this reflex.

The cerebral cortex of normal and treated pups remained undifferentiated into six layers until D28. The outer molecular layer was recognised at D7 because this layer did not contain neurons. Typical pyramidal neurons appear with a perikaryon, and their apical dendrites project towards the pial surface in humans and the monkey, rabbit and guinea pig (Kogan et al., 2000). According to the gradient migration of neurons, cerebral neurons in pups appeared gradually and towards the white matter, following an inside-out migratory pattern.

The most striking features of acrylamide toxicity in the cerebral cortex of the treated group pups were pyknosis and neurocyte chromatolysis, which were observed at all investigated stages. The severity of neurocyte chromatolysis increased with age in group C. This type of brain damage may result from metabolic and biochemical abnormalities caused by acrylamide and its metabolites (freidman, 2003; LoPachin, 2004).

In conclusion, acrylamide adversely affected the neurobehaviour, sensorimotor reflexes and CNS structure of developing rats following maternal acrylamide treatment during the gestation and lactation periods.

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